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(54) Title: MICROBIAL ASSAY

(57) Abstract

A method for the detection of microorganisms, comprises retaining the microorganisms on a substrate and permeabilising the microorganisms without destroying endogenous adenylate kinase, adding ADP, and determining the presence of ATP.

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MICROBIAL ASSAY

Field of the Invention

This invention relates to an assay, and in particular to a rapid microbial counting method.

Background of the Invention

ATP bioluminescence is gaining widespread acceptance in the field of microbiology as a rapid means of detecting microbial contamination. ATP released from microorganisms is made to produce light in a reaction mediated by the luciferase enzyme from fireflies, and the emitted light is detected with great sensitivity by a luminometer. The result is normally displayed as a relative light unit (RLU) value; the higher the figure, the more ATP was present in the sample.

Kits are available commercially for applications such as hygiene monitoring (detecting both microorganisms and organic dirt), presence/absence testing (in which a test sample is incubated in an enrichment broth until any organisms present multiply to detectable levels), and total viable estimation (the RLU value is related to the number of microorganisms present).

The latter approach has drawbacks. It is well known that different microbial species, even in a healthy physiological state, can contain very different amounts of ATP. In addition, when a microorganism is stressed or damaged, as is likely in most test samples, it will have an appreciably lower ATP level. This can lead to considerable inaccuracy when it is attempted to correlate RLU values with microbial numbers measured and with a reference method such as an agar plate count.

Several ways of circumventing this issue have been suggested and two have led to commercial products.

WO-A-9319199 describes a test method in which a filterable test sample, suspected to contain a low number of microorganisms, is divided amongst a series of compartments of equal volume. Enrichment broth is then added to each. After a suitable incubation period, the compartments are assayed individually for microbial growth, by means of ATP bioluminescence. A bioluminescence signal greater than a given threshold value indicates that a compartment originally contained at least one viable microorganism; by comparing the number of compartments giving a positive result with the total, a "most probable number" of organisms in the test sample is calculated. Therefore, this method overcomes the inaccuracy normally associated with ATP bioluminescence by physical division of the test sample, after which a presence/absence test is performed on each sub-sample. The assay is commercially

available from Celsis Limited as the Digital system, which uses a microtitre plate with a membrane filter on the base of each well for the sample division step. The incubation step is at least 24 hours for most sample types, as this is the length of time needed for a single stressed microorganism to recover and multiply to a detectable level. It would be a great advantage for industrial microbiologists to have a "same-shift" (i.e. less than 8 hours) test, as this would allow more timely corrective action if a contamination problem occurs.

A second approach is disclosed in EP-A-0529084. This is based on membrane filtration. Organisms trapped by the membrane are subjected to an ATP bioluminescence assay, using reagents delivered as fine sprays. The membrane comprises a large number of hydrophilic areas separated by hydrophobic partitions so that liquids are confined within the partitions, and the light emitted from a trapped microorganism can be detected as a small spot with an imaging system. The quantification problems associated with ATP bioluminescence are avoided by making use of spatial resolution across the membrane, and the number of light-emitting spots can be related to the viable microbial load of the sample. This is commercially available through Millipore Inc. as the Microstar system. It will count yeast cells directly but bacteria, which contain very much less ATP, require an enrichment step of several hours after filtration, before they can reliably be detected.

ATP bioluminescence is not the most sensitive method for rapid microbial detection. WO-A-9417202 claims the use of adenylate kinase (AK) as a target for microbial assays. This ubiquitous enzyme can be made to convert two molecules of adenosine 5'-diphosphate (ADP) to a molecule each of adenosine 5'-triphosphate (ATP) and adenosine 5'-monophosphate (AMP), after which the ATP can be detected luminometrically using the firefly luciferase reaction. During a reaction time of a few minutes, the microbial adenylate kinase will make a far greater amount of ATP than was present originally in the microorganism; in effect, this method gives an amplification of ATP bioluminescence.

Summary of the Invention

The present invention is based on the finding that the microbial adenylate kinase reaction, and subsequent emission of bioluminescence, can be made to take place on a membrane filter, thereby giving a more quantitative determination of microbial contamination.

According to the present invention, a method for the detection of microorganisms, comprises retaining the microorganisms on a substrate and

permeabilising the microorganisms without destroying endogenous adenylate kinase, adding ADP, and determining the presence of ATP.

Description of the Invention

Preferably, an assay according to the invention comprises the following steps:

(1) Filtration of the test sample through a hydrophilic membrane filter with hydrophobic partitions.

(2) Treatment of the membrane with a reagent containing detergents (to permeabilise microbial cells) and ADP.

(3) Incubation of the membrane, typically for a few minutes, in order to allow production of ATP by the microbial adenylate kinase.

(4) Treatment of the membrane with a luciferase-luciferin reagent.

(5) Analysis of the membrane with an imaging system, to give a count of glowing spots, each of which corresponds to a microorganism or clump of microorganisms in the original sample.

Step (1) is sufficient to ensure that the microorganisms in a sample to be tested are retained on the membrane, during subsequent steps of the process. During subsequent steps, it is desirable to ensure that there is no mixing of the contents of the adjacent zones defined by the partitions.

In some cases, an additional incubation period on a nutrient medium may be required between steps (1) and (2) above: for instance, when the sample contains microbial spores which must be made to germinate before they can be detected, or when extremely stressed or starved microorganisms are present.

The ADP is preferably highly purified. Commercial preparations contain ATP at levels of 0.1% or greater. This may give a high background signal during the bioluminescence step. Therefore, ATP may be removed essentially completely, e.g. by ion-exchange chromatography.

The detergent or detergent mixture should be harsh enough to permeabilise a wide range of microorganisms when they are trapped on a membrane filter, yet not interfere significantly with the activity of adenylate kinase. This can be determined by one of ordinary skill in the art. Suitable detergents are well known. Specific conditions are illustrated in the Examples, and useful/optimum levels of retained AK activity can readily be determined by experiment.

The bioluminescence reagent is preferably essentially free of contaminating adenylate kinase. For example, this is achieved using dye-ligand chromatography.

The bioluminescence reagent preferably provides a steady or slowly-decaying light signal. This can be achieved despite the presence of cell-permeabilising detergents which tend to inactivate firefly luciferase.

The following Examples illustrate the invention.

Example 1

This Example demonstrates that a bioluminescence signal can be generated by the action of adenylate kinase from microorganisms captured on a membrane filter, and that the signal can be used for rapid microbial enumeration. The membrane-handling devices, hand-held nebuliser for reagent delivery, imaging system and image analysis software comprise the Microstar system (Millipore Inc.) which has been designed to detect microorganisms by their ATP content.

Saccharomyces cerevisiae was grown to stationary phase in broth and then diluted in sterile Maximum Recovery Diluent to approximately 160 cfu/ml (determined by parallel agar plate counts). 0.1 ml of this dilution (expected to contain approximately 16 yeast cells) was then added to 20 ml of sterile water and passed through a hydrophilic membrane filter with hydrophobic partitions (Millipore Inc.). The membrane was allowed to dry under sterile laminar flow for approximately 20 minutes, placed in a metal filter holder, and then sprayed with a detergent mixture (Microcount ATP Releasing Agent, Millipore Inc., at double concentration) containing 0.1 mM purified ADP, 10 mM magnesium acetate and 2.5 mM potassium phosphate, pH 6.5.

The membrane was maintained for 15 minutes under laminar flow. During this time, ATP was generated by microbial adenylate kinase. The membrane was subsequently dried and then sprayed with a low-AK bioluminescence reagent and placed in the imaging system. The image was collected for 2 minutes and the number of bright spots calculated by the image analysis software.

The above was performed in duplicate. As a comparison, duplicate membranes were taken through the same procedure but without the inclusion of ADP in the first reagent (so that the assay would detect only the intrinsic microbial ATP, and not the additional ATP generated by the reaction of adenylate kinase). Results were as follows.

Detection Method	Counts Detected
AK	16
	19
ATP	0
	0

Example 2

A suspension of *Escherichia coli* was prepared in maximum recovery diluent. 0.05 ml aliquots were then diluted into 50 ml of sterile water and then immediately passed through 0.45 µm cellulose nitrate membrane filters and placed onto tryptone soya agar plates (for reference plate counts) or passed through membrane filters with hydrophobic grids.

For the adenylate kinase assay, the membrane was allowed to dry under sterile laminar flow, placed in a metal filter holder, and then sprayed with a 1:2 mixture of microbial adenylate kinase assay reagent (MAKAR) and microbial collection and dilution solution (MCDS) (both reagents are available from Celsis Limited). The membrane filter was then placed in a sterile plastic Petri dish which was left in a plastic box containing moist tissue for 20 minutes at 37°C. The membrane was then dried under laminar flow. This procedure ensures that the membrane remains damp during the adenylate kinase reaction step.

After being allowed to dry again under laminar flow, the membrane was sprayed with low-ATP bioluminescence reagent and placed in the imaging system for analysis.

The mean number of spots detected by the adenylate kinase assay, which took less than 1 hour to perform, was 38. The mean plate result for the reference method, counted after 3 days, was 35.

Example 3

A series of 31 purified water samples obtained from 2 pharmaceutical production sites was analysed by the adenylate kinase method and by reference plate counts.

Suitable sample volumes were diluted where necessary into 50 ml of sterile water and analysed by the adenylate kinase method and by reference plate counts. In both cases, the membranes were incorporated into the Milliflex disposable filter

holder system (Millipore Inc.) which allows the funnel to be snapped off after filtration and holds the membrane filter within a plastic ring device.

The adenylate kinase assays were performed as for Example 2 except that the membrane was placed on a Milliflex R2A cassette (Millipore Inc.) at 30°C for 2 hours immediately following filtration. This step aids germination of any spores in the sample and may help to increase the signal from stressed organisms. In addition, the reagents were applied by air-driven spray devices rather than ultrasonic nebulisers. The chosen reference method was incubation for between 5 and 7 days at 30°C on R2A cassettes.

Results are shown in Figure 1. This is a graph of adenylate kinase MPN plotted against reference plate count (RPC). Because there is a tendency at higher microbial levels for more than one organism to be collected in one of the approximately 450 squares formed by the hydrophobic grid, the adenylate kinase results have been converted to MPN values using the formula

$$MPN = -450 \times \ln (1-n/450)$$

where n is the uncorrected number of bright spots counted by the image analysis software.

The graph shows the line of equivalence as a solid line. Clearly there is a high degree of correspondence between the adenylate kinase MPN results, obtained within 3 hours of sample filtration, and the reference plate counts after 5 days or more. It is likely that data points lying significantly above the line of equivalence represent samples containing organisms that could be detected by the adenylate kinase content, but could not grow to visible colonies under the conditions of the reference method.

Example 4

27 of the 31 samples used for Example 3 were tested using a similar adenylate kinase assay method but without the 2-hour recovery step on R2A. The results are shown in Figure 2. The degree of correspondence with the reference method is similar to Example 3, but in this case the adenylate kinase assay took less than 1 hour to perform.

Bacterial spores cannot be detected by the adenylate kinase method until they have been exposed to nutrients and allowed to germinate, but no spore-forming organisms were found in the water samples using conventional identification techniques.

CLAIMS

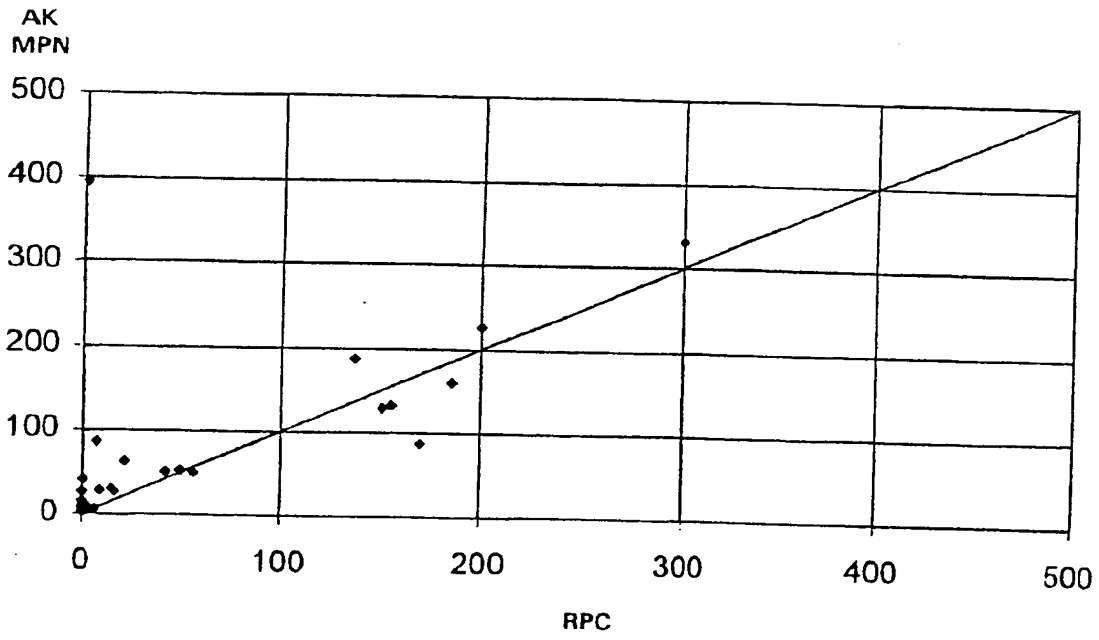
1. A method for the detection of microorganisms, which comprises retaining the microorganisms on a substrate and permeabilising the microorganisms without destroying endogenous adenylate kinase, adding ADP, and determining the presence of ATP.

2. A method according to claim 1, wherein the determining is by bioluminescence.

3. A method according to claim 1 or claim 2, wherein the added ADP is essentially free of ATP.

4. A method according to any preceding claim, wherein the microorganisms are present in one or more discrete areas of an assay device.

5. A method according to any preceding claim, wherein the substrate is a membrane filter having zones defined by hydrophobic partitions.


AK
MPN
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400
300
200
100
0
0
100
200
300
400
500
RPC

Fig. 1

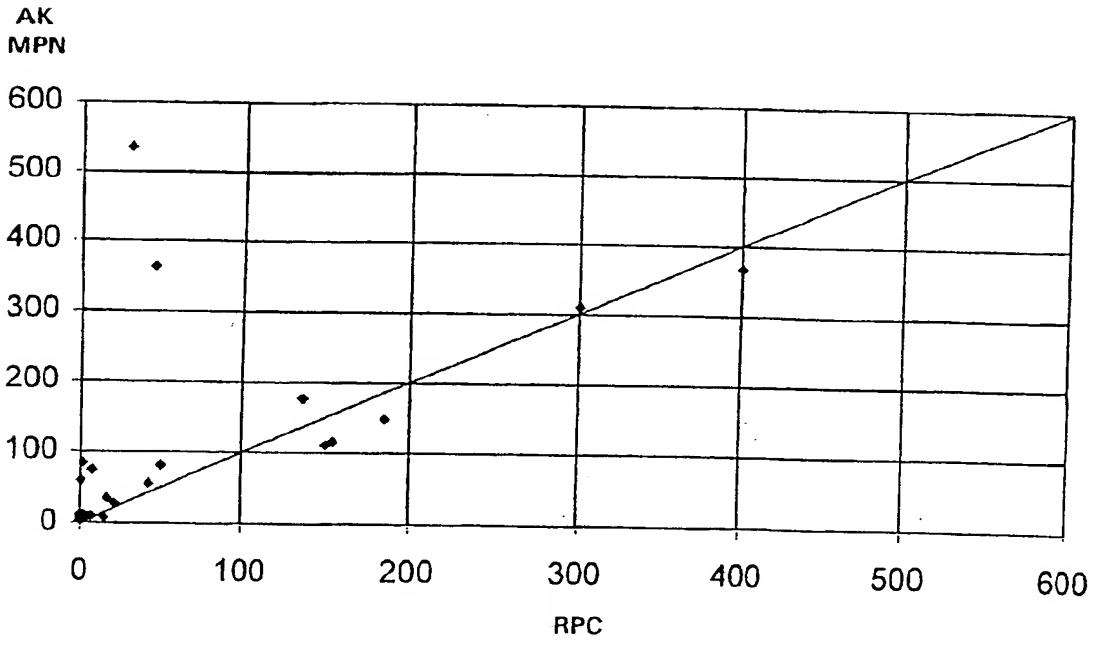
AK
MPN
600
500
400
300
200
100
0
0
100
200
300
400
500
600
RPC

Fig. 2

INTERNATIONAL SEARCH REPORT

Int tional Application No
PCT/GB 99/00438

A. CLASSIFICATION OF SUBJECT MATTER
IPC 6 C12Q1/04 C12Q1/48

According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)
IPC 6 C12Q

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practical, search terms used)

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category °	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
X	WO 96 02666 A (SECR DEFENCE ;SQUIRRELL DAVID JAMES (GB)) 1 February 1996 see the whole document	1-5
A	WO 96 02665 A (SECR DEFENCE BRIT ;SQUIRRELL DAVID JAMES (GB)) 1 February 1996 see the whole document	1
A	WO 94 17202 A (SECR DEFENCE BRIT ;SQUIRRELL DAVID JAMES (GB)) 4 August 1994 cited in the application see examples	1

☐ Further documents are listed in the continuation of box C.

☒ Patent family members are listed in annex.

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INTERNATIONAL SEARCH REPORT

Information on patent family members

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